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Effects of fluctuant magnesium concentration on phenotype of the primary chondrocytes

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Abstract: Magnesium (Mg) and its alloys have attracted much research interest as degradable implant materials. Mg ions may enhance phenotype of chondrocytes at optimal concentrations. In this study, we investigated the effects of fluctuant concentrations of Mg ion released from *in vitro* degradation of pure Mg microspheres on the phenotype of chondrocytes. The chondrocytes were cultured with 250 μg/mL, 500 μg/mL, and 1000 μg/mL of Mg microspheres (75–150 μm) either on tissue culture plates or within alginate hydrogels, with 5, 10, and 20 m*M* of $MgCl_2$ solution set as the control group. Concentrations of Mg ions and pH values of the culture medium were measured at 3 days' interval. Cytotoxicity was evaluated while glycosaminoglycan (GAG) content and gene expression of collagen type I/II/X, aggrecan were quantified. Results showed that peak concentrations of Mg ion reached 10, 20, 30 m*M*, respectively, at day 3 in groups containing Mg-250 μg/mL, Mg-500 μg/mL, and Mg-1000 μg/mL, respectively, whereas pH values increased mildly to approximately 8 in all experimental groups. No significant cytotoxic effects were found at day 1 and day 3 in all experimental groups. GAG content increased 6% at day 14 in Mg-250 μg/mL group in tissue culture plate, but not in the hydrogel culture. Gene expression of collagen type I/II/X and aggrecan in Mg-1000 μg/mL group decreased significantly when chondrocytes were cultured in cell culture plates. Increase of gene expression of collagen type X in Mg-250 μg/mL group at day 7 was observed. However, gene expressions of collagen type I/II/X and aggrecan in Mg groups increased significantly at day 7 when chondrocytes were cultured in hydrogels. It was concluded that the phenotype of chondrocytes was regulated with dynamic concentration of Mg ions and pH values in a dose- and time-dependant manners. Fine-tuned degradation of Mg microspheres could be used to facilitate layered structures of articular cartilage. Furthermore, it would be cautious to extrapolate from results from 2D chondrocyte cultures. © 2014 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2014.

Key Words: magnesium, degradation, chondrocyte

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INTRODUCTION

Magnesium (Mg) and its alloys have shown great potential as biodegradable materials, such as vascular stents and bone substitutes.[1–4] Mg ions increase alkaline phosphatase (ALP) activity of osteoblasts and facilitate osteogenic differentiation and subsequently increase bone mass and integration with host bone.[5–8] Mg alloys also have been used as cardiovascular stents because of their excellent mechanical properties and ductility. Mg alloys generally degrade to magnesium hydroxide, hydrogen gas, sulfate, and phosphate in aqueous environments via electrochemical corrosions, especially with high concentration of chloride ions.[9] Mg ions and OH^- ions are released into the fluids subsequently when the magnesium hydroxide film breaks down.[10] These dynamic processes have significant effects on the surrounding tissues. Multiple factors are involved in degradation of Mg alloys, such as alloying elements, manufacturing processes, change of pH values, ion flow, and thermal effects.[11–13] Therefore, many strategies have been adopted with an aim to control degradation of Mg and its alloys, including surface modification, alloying with other elements, and hydrogel encapsulation.[14–18]

Mg ions are important for both the development and maintenance of cartilage at dose-dependent modes. Mg depletion or a Mg-deficient diet leads to cartilage lesions during cartilage development.[19,20] Injections of magnesium sulfate ($MgSO_4$) into intra-articular have shown to reduce experimental osteoarthritis and nociception through

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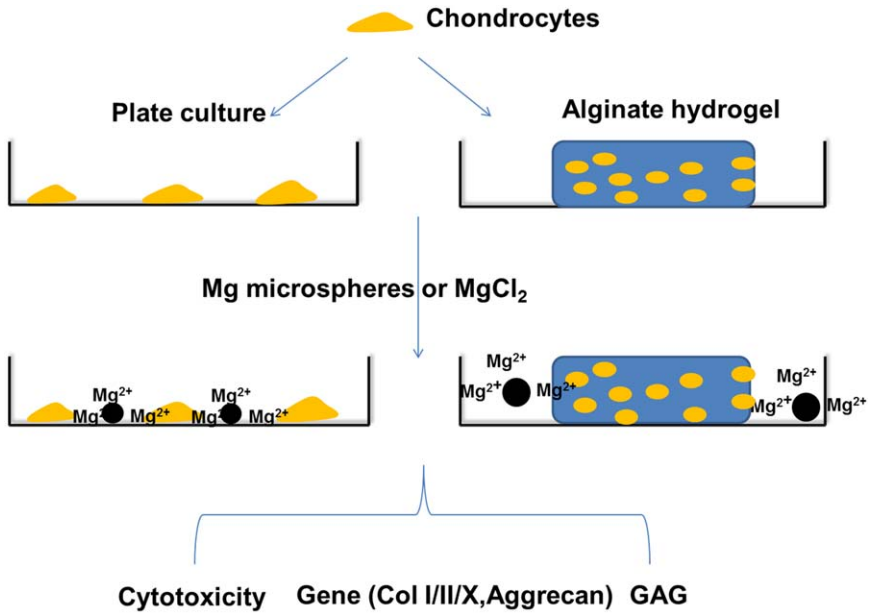

FIGURE 1. Exprimental design. Primary chondrocytes expanded 2 weeks in plate culture, and then were seeded into plate or hydrogel, respectively. Yellow dots indicate the chondrocytes, whereas black dots indicate Mg microspheres. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

inhibition of the NMDA receptor.[21] Mg ions have also shown to increase adhesion of human synovial mesenchymal stem cells (MSCs) through activation of integrins and subsequently enhances synthesis of extracellular matrix *in vitro* and *in vivo*.[22] Previous study showed that 10 m*M* of Mg ion concentrations could enhance proliferation of human articular chondrocytes, whereas 20 m*M* of Mg ion concentration decreased cell proliferation and improved cell differentiation evidenced by an increase in gene and protein expression of aggrecan and collagen type II.[23,24] Articular cartilage is composed of four layers, namely superficial, intermediate, deep, and calcified zones, whereas chondrocytes embedded in the individual zones have varying phenotypes.[25] The chondrocytes in the superficial zone express abundant collagen type I, whereas chondrocytes in the intermediate zone express more cartilage oligomeric protein. Both collagen type IX and X exist in the middle and deeper zones. Therefore, different concentrations of Mg ion may have varying effects on the phenotypes of chondrocytes.

Effects of Mg on chondrocytes have been investigated in previous studies; however, all these reported studies adopted static concentration instead of dynamic ones. Previously, the cells were either seeded directly onto the surface of bulk alloys or cultured in the medium containing $MgCl_2$ or alloy extraction. It did not necessarily mimic the dynamic *in vivo* Mg alloy degradation processes.[23,24,26] The effects of dynamic degradation of Mg alloys on the phenotypes of chondrocytes remain elusive. Degradation of Mg alloys leads to changes of both Mg ion concentrations and pH values of the surrounding aqueous environment with time. Released nanoparticles may lead to dose-dependent cytotoxicity and genotoxicity.[27] In this study, pure Mg microspheres (75–150 μm) were co-cultured with pig chondrocytes, which cultured in plate (2D) or alginate hydrogel (3D) to simulate dynamic degradation of Mg *in vivo*, whereas 5, 10, and 20 m*M* $MgCl_2$ were set as controls (Fig. 1). We evaluated the dynamical concentrations of Mg ion released from Mg-250 μg/mL, Mg-500 μg/mL, Mg-1000 μg/mL microspheres and pH values in correlation with cytotoxicity, GAG deposition, and expression of key genes to explore the applications of Mg in cartilage or osteochondral regeneration.

MATERIALS AND METHODS

Fabrication of Mg microspheres

Pure Mg microspheres with purity of 99.80 wt % were manufactured with atomization method (Tangshan Weihao Magnesium Powder Co., China). Diameters of the microspheres ranged between 75 and 150 μm. Microspheres (75–150 μm) of pure Mg were used in all experiments, respectively.

Isolation and culture of the primary chondrocytes

Animal study was approved by the ethics committee of Fuwai Cardiovascular Hospital (Beijing, P.R. China). Cartilage tissues were isolated from knee joints of Wuzhishan miniature pigs (1 year old, male/female) within 12 h post-sacrifice. The chondrocytes were harvested through enzymatic digestion with 0.2% of type II Collagenase (17101-015, Gibco-Invitrogen) at 37°C for 6 h. The cells were filtered and centrifuged before culturing in monolayers at a density of 20,000 cells/cm^2 in 100 mm plate (430167, Corning) in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (SV30087.02, Hyclone), 100 U/mL of penicillin/and 100 μg/mL of streptomycin at 37°C with 5% CO_2. Medium was changed at 3 days' intervals. The cells were trypsinized until 85–90% confluence

TABLE I. Primer Sequences Used in Gene Expression Analysis

Gene	Primer Sequence (5′-3′)
GAPDH forward	GTCATCCATGACAACTTCGG
GAPDH reverse	GCCACAGTTTCCCAGAGG
Col I forward	CAGAACGGCCTCAGGTACCA
Col I reverse	CAGATCACGTCATCGCACAAC
Col II forward	GAGAGGTCTTCCTGGCAAAG
Col II reverse	AAGTCCCTGGAAGCCAGAT
Col X forward	CAGGTACCAGAGGTCCCATC
Col X reverse	CATTGAGGCCCTTAGTTGCT
Aggrecan forward	CGAAACATCACCGAGGGT
Aggrecan reverse	GCAAATGTAAAGGGCTCCTC

was achieved and passage 2 chondrocytes were used for the following studies.

Culture of chondrocytes in 2D or 3D with Mg microspheres

Mg microspheres were sterilized with UV radiation for 1 h. In 2D culture, $1 \times 10^4/cm^2$ chondrocytes were seeded onto 6-well plates 12 h before 250, 500, and 1000 μg/mL of Mg microspheres were added into each well. In 3D culture, cells were encapsulated in alginate hydrogel. In brief, 50 μL of medium containing cells (2×10^6/mL) was added into 200 μL of 1.2% alginate solution dissolved in phosphate buffer solution (PBS) and mixed uniformly, before 300 μL of 100 m*M* $CaCl_2$ solution was mixed. 5, 10, and 20 m*M* $MgCl_2$ culture medium were used as controls. The cells/microspheres systems were co-cultured at 37°C with 5% CO_2, whereas medium was changed after 3 days' time interval.

Concentrations of Mg ions and pH value of culture medium

Concentrations of Mg ion in medium with microspheres were measured at days 1, 3, and 7 with inductively coupled plasma atomic emission spectrometry (ICP-AES; Leeman, Profile ICP-AES). The pH values were measured using a pH meter.

FDA-PI staining and scanning electron microscopy

The cells were incubated with Fluorescein diacetate and Propidium iodide (FDA-PI). Samples were rinsed in PBS before incubated with 2 μg/mL of FDA at 37°C for 15 min. The samples were further incubated with 5 μg/mL PI for 5 min after rinsing with PBS. The samples were visualized under confocal laser scanning microscope (CLSM, LSM510, Zeiss, Germany) at 494 nm (green) and 540 nm (red), respectively.

Cell/microspheres constructs were rinsed with PBS and fixed in 2% glutaraldehyde solution for 2 h at room temperature before dehydrated in a gradient ethanol/distilled water mixture. The microspheres were sputter-coated with gold and were observed using scanning electron microscopy (SEM; Quanta 200FEG, FEI).

Cytotoxicity

MTT assay (3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) (MTT, 2128, Sigma) was performed at day 1 and 3, respectively. About 500 μL of MTT solution (5 mg/mL) was added in each well and incubated at 37°C for 3 h. All wells were emptied before 2 mL DMSO was added. Optical density was measured at 570 nm wavelength using a plate reader (Bio-Rad). The value was compared and recorded as percentages after subtracting the data using the blank control. Three replicates of each sample were used.

GAG quantification

Amounts of GAG were measured as reported previously.[26] In brief, cells were digested in 1 mL of 50 μg/mL of proteinase K (Beijing Hualvyuan Biotechnology Development Center, P.R. China) at 56°C overnight, before 0.5 mL of Dimethylmethylene Blue (DMMB, 341088, Sigma) solution was added and vortexed for 30 min. The samples were centrifuged and re-dissolved in 0.5 mL of decomplexation solution and vortexed for 30 min, before optical densities were measured at 630 nm. Standard curves of sGAG content were recorded with chondroitin sulphate (27042-10G-F, Sigma). The assay was performed in triplicates.

Real-time PCR

Cells were lysed in 1 mL Trizol (15596-026, Invitrogen) for 5 min. The total RNA was extracted following the manufacturer's instructions. RNA concentrations were determined with NanoDrop. cDNA synthesis was performed using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA) following manufacturer's instructions. Real-time PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA) on Applied Biosystem 7500 Real-Time PCR System (Applied Biosystem) at 95°C for 15 min, followed by 40 cycles of 15-s denaturation at 94°C, 30-s annealing at 55°C, and 30-s elongation at 72°C. The target genes were normalized to the reference gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Gene expression was calculated as $2^{-\Delta\Delta Ct}$. Experiments were

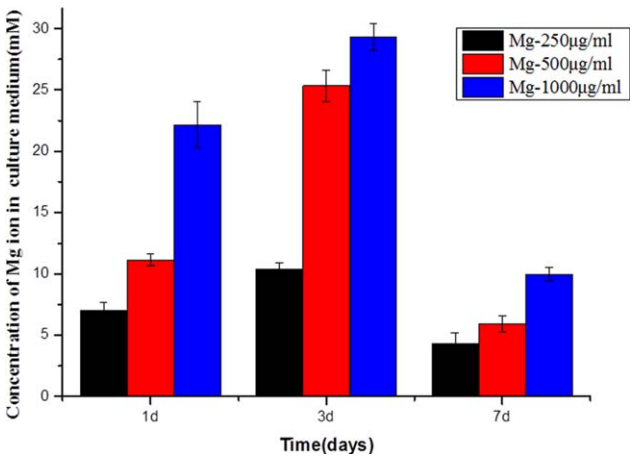

FIGURE 2. The change of Mg ion concentration in the cell culture medium as a result of degradation of different concentration Mg microspheres or added $MgCl_2$ in culture medium at day 1, 3, and 7. Mg ions include ions from original media (about 0.8 m*M*). Values are mean ± SD, $N = 3$. Note: mg/L = ppm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE II. Magnesium Ion Concentration Released From Different Concentration Microspheres and pH Value of Culture Medium

	Mg- 250 μg/mL	Mg- 500 μg/mL	Mg- 1000 μg/mL
Highest concentration on day 3 (m*M*)	10.4 ± 0.5	25.33 ± 1.2	29.33 ± 1.1
Average concentration before 3 days (m*M*)	6.96	13.3	20.08
pH value on day 3	7.7 ± 0.12	8.15 ± 0.18	8.35 ± 0.2

performed in triplicates for each gene. The primers are listed in Table I.

Statistics

Statistical analysis was performed using SPSS 13.0 (One-way ANOVA, LSD, $p < 0.05$). Error bars on all graphs are expressed as mean ± standard deviation (SD), where $n = 3$.

RESULTS

Concentration of Mg ion and pH change of culture medium

Concentrations of Mg ions in the culture medium gradually increased with degradation of the Mg microspheres, whereas rates of degradation decreased with time in all experimental groups (Fig. 2). Peak concentrations of Mg ion were recorded at day 3 before medium change, 10.4 ± 0.5 m*M* (Mg-250 μg/mL group), 25.3 ± 1.2 m*M* (Mg-500 μg/mL group), 29.3 ± 1.1 m*M* (Mg-1000 μg/mL group), whereas average concentrations were 6, 12, and 20 m*M*, respectively (Table II).

pH values of the culture medium increased from 7.2 to 8.35 with both time and increasing amount of the Mg alloys (Fig. 3). pH values of the culture medium recorded were 7.7 (Mg-250 μg/mL group), 8.15 (Mg-500 μg/mL group), and 8.35 (Mg-1000 μg/mL group) at day 3, respectively. pH value kept no more than 8 at day 7 in all experimental groups.

Cytotoxicity and morphology of Mg microspheres and cells

Cell viabilities were approximately 80% at day 1 in all experimental groups (not shown). However, there were no significant differences in individual experimental groups and blank control group at day 3; however, cell viability of $MgCl_2-20$ m*M* group was less than 50% in the plate culture at day 3 (Fig. 4).

The chondrocytes had spindle like morphology and there was no significant difference in cell morphology between individual experimental groups in plate culture. The results showed that the chondrocytes proliferated with time while some of them integrated with the Mg microspheres in the experimental groups [Fig. 5(A–C)]. Surface of the microspheres cracked showing signs of degradation [Fig. 5(D,E)]. The confocal microscopy micrographs showed cell attachment onto the surface of the Mg [Fig. 5(F)].

GAG quantification

In plate culture, GAG content in all experimental groups increased between day 7 to day 14. There was no significant difference between individual experimental groups at day 7; however, GAG content increased up to 6% in Mg-250 μg/mL group, whereas decreased 40% in Mg-1000 μg/mL at day 14 [Fig. 6(A)]. In hydrogel culture, GAG contents decreased mildly at day 14 in all three experimental groups, but there was no significant difference between experimental groups and blank control. GAG content of $MgCl_2-10$ m*M* and $MgCl_2-20$ m*M* group decreased significantly compared to the blank control at day 14 [Fig. 6(B)].

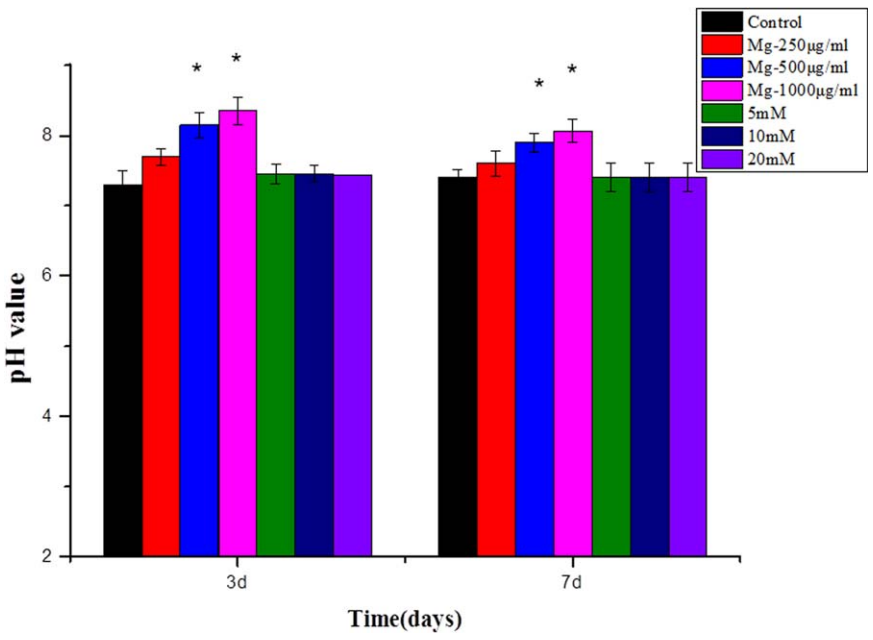

FIGURE 3. The pH value change of different culture medium at day 3 and day 7. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

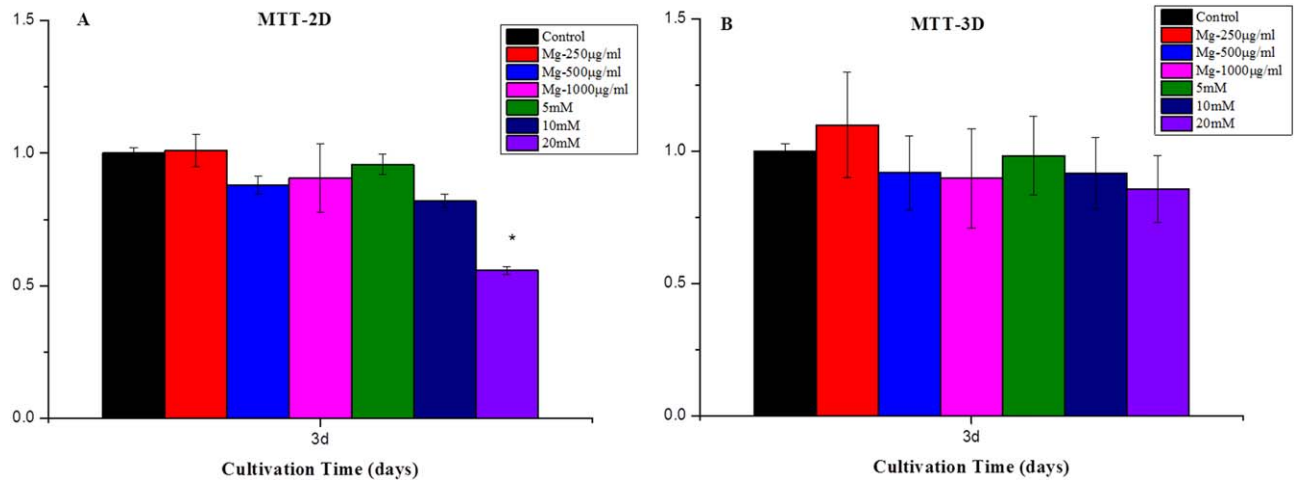

FIGURE 4. Cytocompatibility of different concentration of $MgCl_2$ and Mg microspheres at day 3 by the method of MTT test. (A) plate culture, (B) hydrogel culture. $*p < 0.05$ compared to bland control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RT-PCR

For collagen type I/II and aggrecan, there were no significant difference between experimental groups and the blank control. However, the gene expression of collagen type X was up-regulated significantly 1.8–4.0 folds in the experimental groups with dose-dependent mode in plate culture at day 3 [Fig. 7(A)]. Gene expression of collagen type I/II and aggrecan decreased in all experimental groups (except for Col I, Mg-250 μg/mL group) with a dose-dependent mode at day 7. Significant difference in values

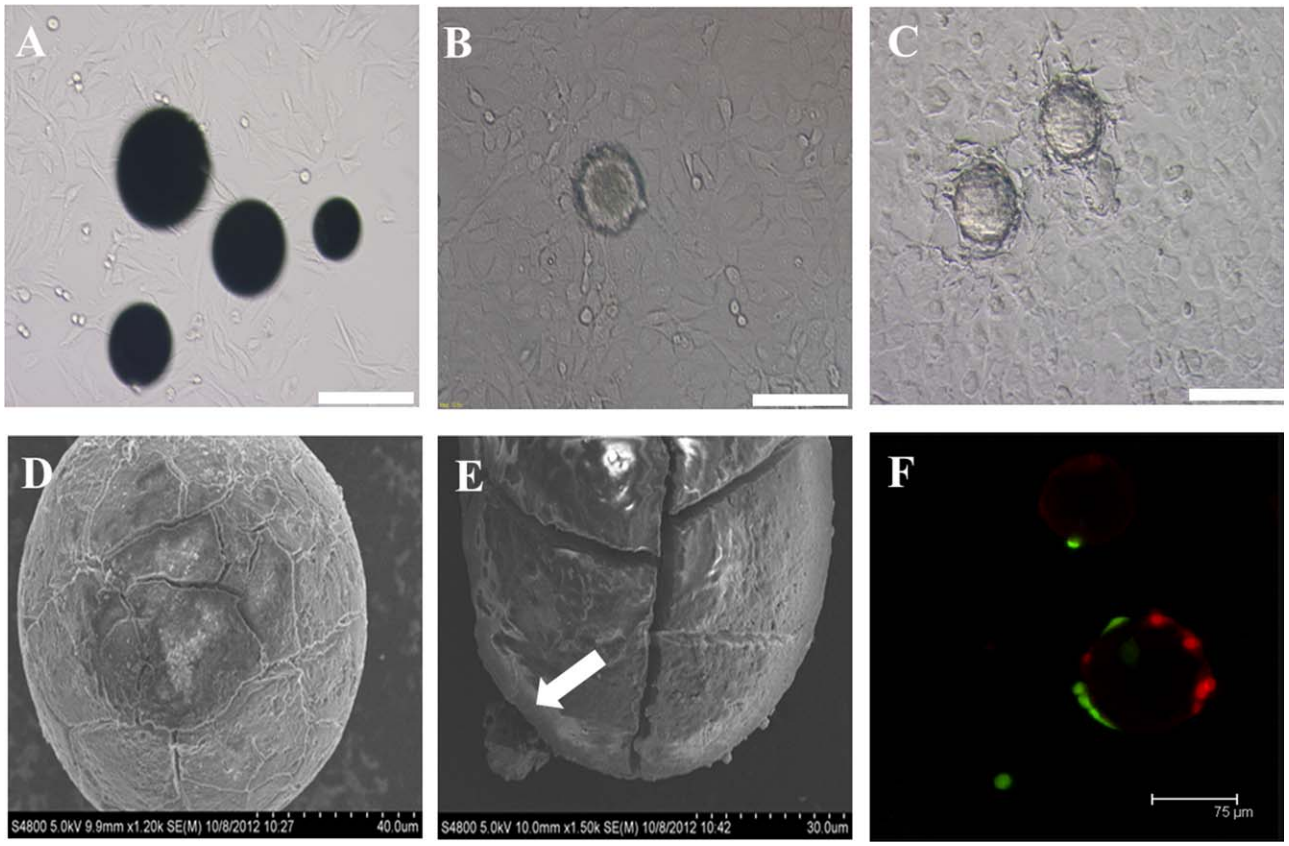

FIGURE 5. Morphology of alloy microspheres and chondrocytes: (A–C) optical microscope of Mg-250 μg/mL group at day 3, 7, 14, respectively; (D–E) scanning electron micrographs (SEM) of microspheres and cells on the surface after degradation 7 days; (F) laser scanning confocal microscopy of cells grown on the surface of the Mg microspheres at day 7. White scale bars in Figure 4(A–C) = 100 um. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

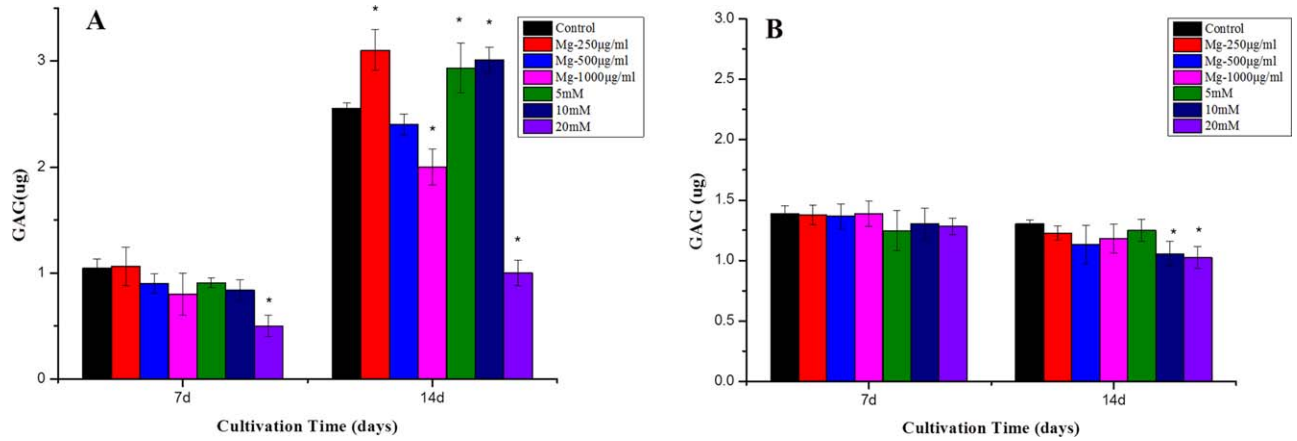

FIGURE 6. GAG secretion of chondrocytes cultured in different concentration microspheres or $MgCl_2$ at day 7 and day 14. (A) plate culture, (B) hydrogel culture $^*p < 0.05$ compared to blank control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

were found in all aggrecan groups, some collagen II groups (Mg-500 μg/mL ($p = 0.03$) and Mg-1000 μg/mL groups ($p = 0.02$)) and one collagen I groups (Mg-1000 μg/mL). Gene expression of collagen X increased significantly ($p = 0.02$) in Mg-250 μg/mL group at day 7.

Gene expression of Col I/II/X and aggrecan kept stable at day 3, whereas increased significantly at day 7 in the hydrogel culture. Gene expression of collagen type II increased significantly with an increasing amount of Mg, whereas no similar trends for other gene expressions were recorded [Fig. 7(B)].

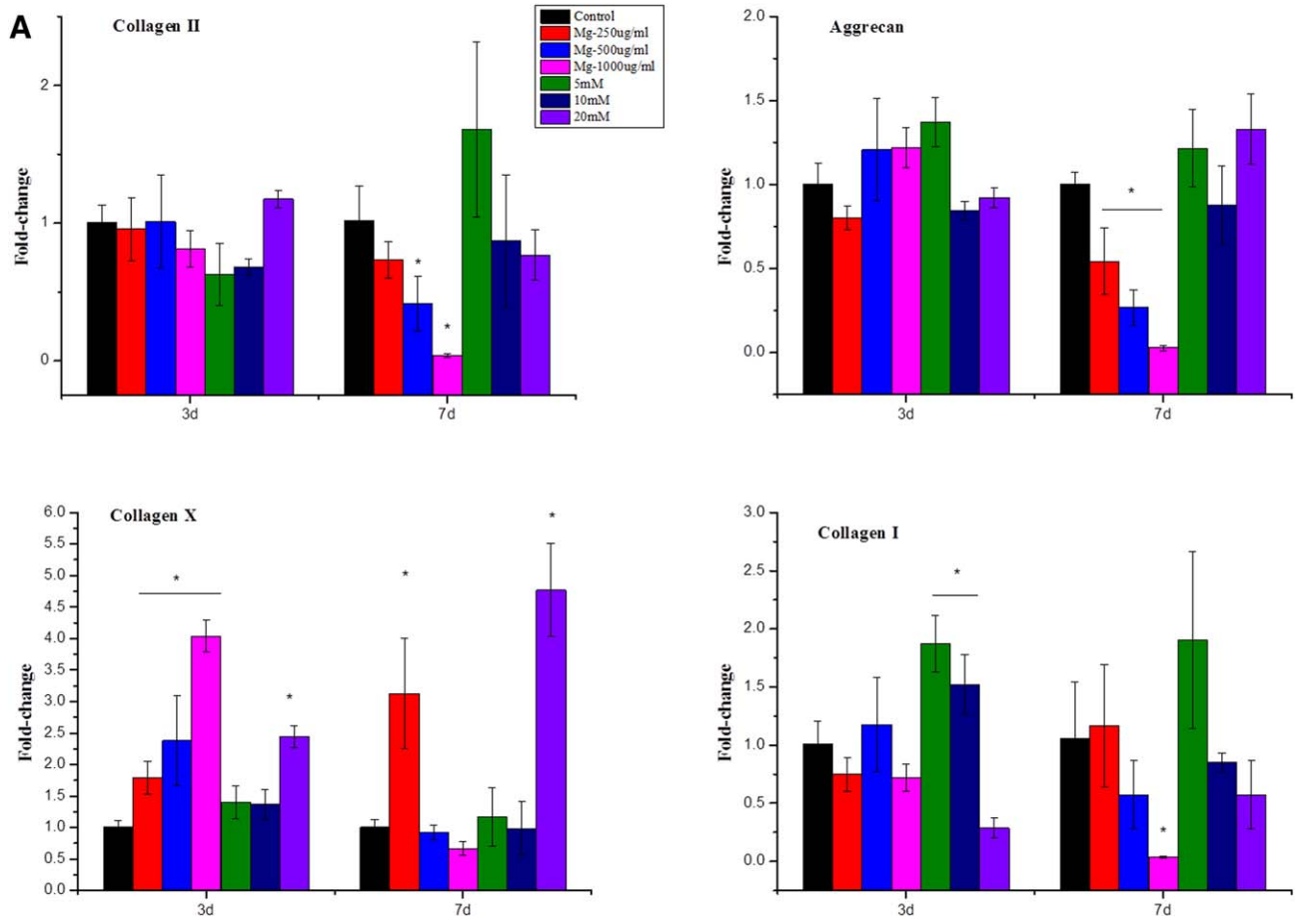

FIGURE 7. Gene expression of chondrocytes at day 3 and day 7. (A) plate culture, (B) hydrogel culture. $^*p < 0.05$ compared to blank control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

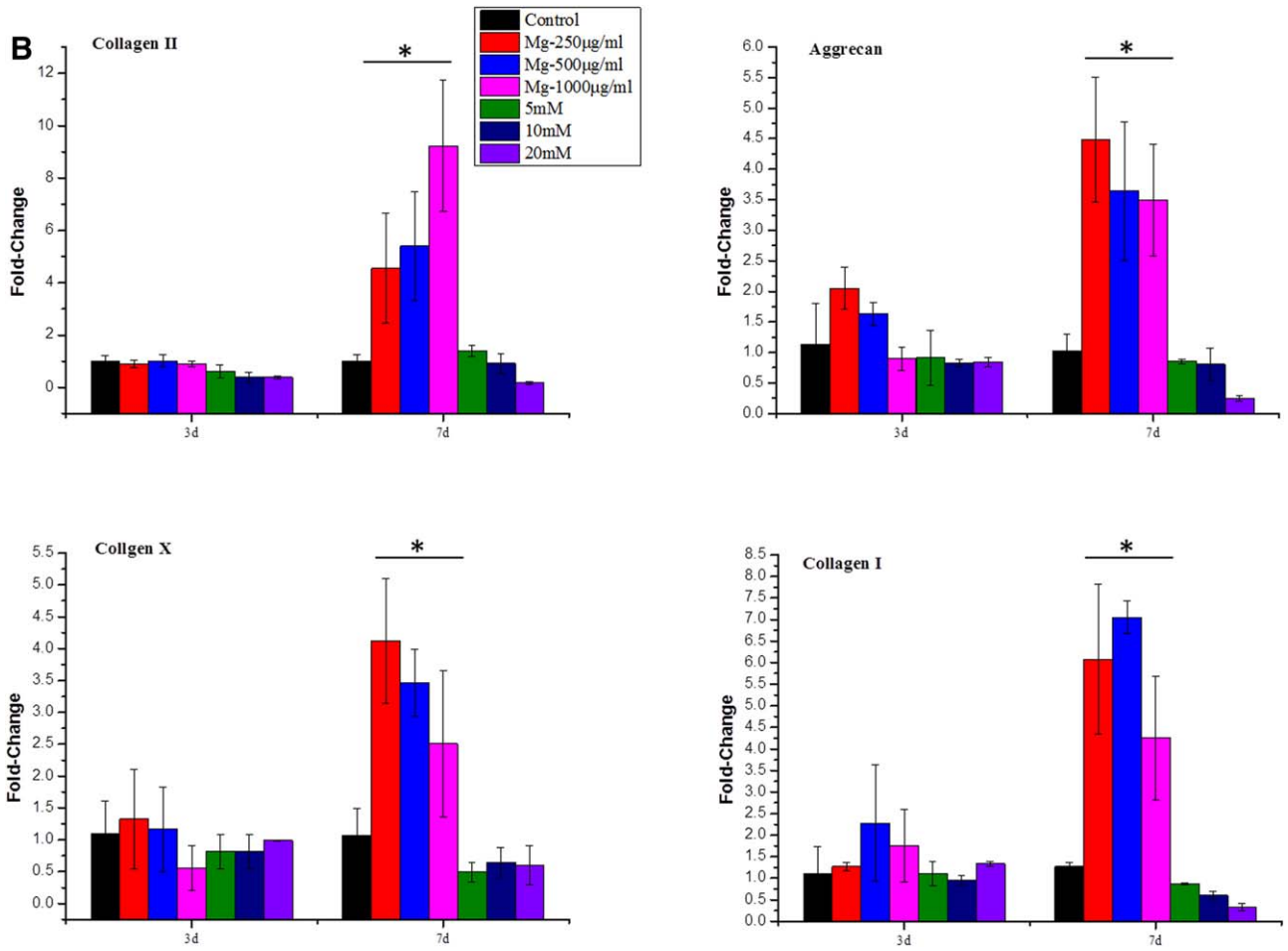

FIGURE 7. Continued

DISCUSSION

Dynamic concentrations of Mg alloys have an effect on chondrocytes, whereas both pH values and degradation particles of Mg are involved.[27–29] Different concentration of Mg ions showed varied effects on the phenotype of chondrocytes.[23] Low concentration of Mg (10 m*M*) enhanced chondrocyte proliferation, whereas high concentration of Mg (20 m*M*) hindered chondrocyte proliferation while enhanced expressions of both gene expression and protein expression of collagen type II. Similarly, high concentration of Mg ion improved chondrogenic differentiation of mesenchymal stem cells through enhancing the function of integrins.[22] Previous studies demonstrated that deficiency of Mg ion degenerated cartilage through hindering function of integrins whose functions can be effected by the divalent cations.[20] Studies have shown that the supplement of Mg ion in Al_2O_3 increased adhesion of osteoblasts to implants via integrins.[27] In addition, size of the Mg microspheres and related degradation particles could change the local microenvironment significantly.[28] In this study, 500 μg/mL 325 mesh (<40 μm) Mg microspheres were toxic and therefore majority of dead cells were seen at day 1 whereas using the same concentration of larger microspheres (75–150 μm) showed no signs of cytotoxicity (data not shown). It could be reasonably attributed to the abrupt changes of Mg ion concentration and pH value in 325 mesh microspheres or nanoparticles broken down from microspheres due to degradation. Relatively small Mg microspheres (325 mesh) showed cytotoxic and genotoxic effects even at low concentration (25–100 μg/mL) on rat osteosarcoma.

There are four cell layers in cartilage with different phenotypes.[25] Varying amounts of Mg led to significantly different gene expressions.[30–32] It could be beneficial for differential induction of chondrocytes into subpopulations in different cartilage layers. Potentially, larger amount of Mg could be used to induce higher collagen type II expression in the superficial and middle layers, while relatively low concentration could be used to promote collagen X synthesis of deep zone. A even higher amount of Mg could be used to enhance phenotypes of osteoblasts and cells in the calcified cartilage zone. Certainly, aggrecan and other genes should be fine-tuned as a whole.

Culture conditions have a significant effect on the phenotypes of chondrocytes. In hydrogel cultures, the effect of Mg microspheres on the chondrocytes differed from those in the plate cultures. The chondrocyte phenotype differed when cultured on the tissue culture plates or hydrogels. Firstly, chondrocytes dedifferentiate during *in vitro*

expansion when cultured on the plate culture with significant changes, including cell elongation, formation of actin stress fibers, and production of collagen type I.[25] When cultured in alginate hydrogels, the previously dedifferentiated chondrocytes could re-differentiate.[25] Secondly, the alginate could prevent the cells from contacting the microspheres directly and buffer potentially abrupt changes of microenvironments.[33] The last but not least, concentrations of Mg ion released from microspheres could be different for the chondrocytes in hydrogels, compared with the cells cultured on the culture plates.

Ideally, dynamic Mg concentrations derived from degradation of Mg alloys should enhance chondrogenesis, osteogenesis, and integration of de novo cartilage and bone. To achieve this target, well-controlled degradation of Mg alloys should be fine-tuned via various modifications, such as surface pre-treatment, polymer mixture, and so on. Possibly, there were even larger fluctuation of pH values and ion concentrations in peripheral areas of degrading Mg than these measured using the medium at relatively large intervals, which may affect the cell viability. In the meantime, *in vivo* environments are tissue-specific for Mg degradation, which further adds complexity to the challenge at hand. For further clinical applications, Mg particles can be integrated into biomaterials to control the release of Mg ion as therapeutic agents.

CONCLUSIONS

Degradation of Mg alloys dynamically changed the physiological environment, especially concentration of Mg ions and pH values. The results were significantly different from those recorded by $MgCl_2$. In 2D culture, low concentrations of Mg ions enhanced excretion of extracellular matrix, whereas extra-high concentration of Mg inhibited the gene expression. In 3D culture, degradation of Mg microspheres increased the gene expression of chondrocytes dose-dependently. Dynamic degradation of Mg could be harnessed to regenerate cartilage with varying layers and osteochondral grafts as an ion therapeutic agent.

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